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ON THE ROLE OF Ca^{2+} IN THE TRANSMITTER CHOICE MADE BY CULTURED SYMPATHETIC NEURONS[1]

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Abstract

Depolarization or neuronal activity influences the differentiation of neonatal rat sympathetic neurons in dissociated cell culture by reducing their ability to respond to a cholinergic factor in conditioned medium (CM), allowing adrenergic differentiation to proceed (Walicke, P., R. Campenot, and P. Patterson (1977) Proc. Natl. Acad. Sci. U. S. A. *74:* 5767–5771). The present study analyzes the role of Ca^{2+} in the mechanism of this effect of activity. Addition of the Ca^{2+} influx inhibitors, $MgCl_2$, D600, diphenylhydantoin (DPH), or EGTA to the growth medium overcomes the developmental effect of depolarization. Elevation of the $CaCl_2$ level in the medium, or addition of $BaCl_2$, slightly enhances the effect of depolarization. In non-depolarized cultures, Ba^{2+} potently inhibits cholinergic differentiation and the additional Ca^{2+} has a similar, though smaller, effect. Chronic depolarization of the neurons with either elevated K^+ or veratridine leads to an increase in cyclic AMP (cAMP) content, and this elevation is blocked, along with the effect on transmitter choice, by $MgCl_2$ and D600. On the other hand, EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid) and DPH, which also favor cholinergic differentiation, have little effect on the depolarization-induced elevation in cAMP. Exogenous addition of cyclic nucleotide derivatives or prostaglandin E_1, like depolarization, decrease cholinergic induction. Addition of EGTA, however, decreases the developmental effects of these agents while not interfering in their ability to increase cAMP levels. Thus, there are several ways cAMP levels can be uncoupled from the transmitter choice.

The ability of the Ca^{2+} influx inhibitors to counteract the effect of depolarization on development and cAMP levels probably does not reflect the blockade of an action of an interposed neurotransmitter, since addition of phentolamine, phenoxybenzamine, adenosine deaminase, or alloxazine have no effect on transmitter choice or cAMP levels. Although calcium and cAMP undoubtedly have complex interactions in these neurons, calcium appears to play a major role in the developmental effects of depolarization and in the intracellular events governing transmitter choice.

Electrical activity is an important developmental signal for neonatal sympathetic neurons *in vivo* (Black et al., 1971) and *in vitro* (Walicke et al., 1977). The accompanying paper deals with the possible role of cyclic adenosine 3′:5′-monophosphate (cAMP) as a second messenger in the effects of activity on development (Walicke and Patterson, 1981). Although exogenous cAMP mimics the developmental effects of depolarization and depolarization increases intracellular cAMP, not all of the available evidence supports the cAMP hypothesis. For example, theophylline inhibits the ability of depolarization to enhance adrenergic differentiation while still allowing an increase in cAMP (Walicke and Patterson, 1981).

Alterations in internal ionic composition in response to activity may be another mechanism by which depolarization could affect neuronal development. Sympathetic neurons have voltage-sensitive Ca^{2+} channels in their somata which allow Ca^{2+} entry during firing of action potentials (Horn and McAfee, 1980; McAfee and Yarowsky, 1979; O'Lague et al., 1978). Depolarization with either elevated K^+ or veratridine increases Ca^{2+} influx in a variety of neuronal preparations (Blaustein, 1976; Goddard and Robinson, 1976; Robinson and Kabela, 1977; Stallcup, 1979). Thus, Ca^{2+} seems a good candidate for mediating the effects of depolarization on neuronal transmitter choice.

Furthermore, it is well recognized that Ca^{2+} plays an important role in the control of numerous cellular events, sometimes in association with cyclic nucleotides (Rasmussen and Goodman, 1977). Calcium and possibly calmodulin play an important role in a number of neuronal

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functions, including transmitter release and axonal transport (Cheung, 1980; Means and Dedman, 1980). In addition, Ca^{2+} can influence cellular cyclic nucleotide metabolism through activation of adenylate cyclase, guanylate cyclase, and phosphodiesterase. Furthermore, the brain contains large amounts of a Ca^{2+}-dependent protein kinase which is similar to cyclic nucleotide-dependent kinase and whose function is largely unknown (Cheung, 1980; Means and Dedman, 1980). These findings suggest that Ca^{2+} could affect the developmental choices of sympathetic neurons. The involvement of Ca^{2+} provides a mechanism through which neuronal activity could be directly linked to long term changes in neuronal development. This paper examines the influence of a variety of Ca^{2+} effectors on transmitter choice in cultured sympathetic neurons.

Materials and Methods

Superior cervical ganglia were removed from neonatal rats, mechanically dissociated into single cells, and maintained in tissue culture as previously described (Walicke and Patterson, 1981). Culture medium, conditioned medium (CM), and ammonium sulfate precipitates of CM were all prepared and handled as previously described (Walicke and Patterson, 1981). Twenty and 53 mM K^+ media were prepared from special Na^+- and K^+-free Leibowitz L15 medium obtained from Grand Island Biological Co. (Walicke et al., 1977).

For additions of Ca^{2+}, Ba^{2+}, Sr^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}, solutions of the Cl^- salts were made up at concentrations in the molar range and added to the medium; osmolarity was not corrected. Stock solutions of sodium ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) were made up with the pH adjusted to 7.4 and were filtered through a 0.2-μm pore size Millipore filter for sterilization. pH adjustments were made with 1 M NaOH using the indicator dye in the medium. D600 was obtained from Knoll Chemische Fabriken, Germany. All other chemicals were obtained from Sigma.

Transmitter synthesis was assayed after 4-hr incubations with [^{3}H]tyrosine and [^{3}H]choline as previously described (Walicke and Patterson, 1981). L15-CO_2 medium without added pharmacological agents was used for all assays. Protein and cyclic nucleotide assays also were performed as previously described (Walicke and Patterson, 1981).

Results

K^+ depolarization and calcium. The effects of Ca^{2+} availability on the change in transmitter choice caused by K^+ depolarization were examined by adding agents which interfere with Ca^{2+} influx. These included 20 mM Mg^{2+}, 2 mM Mn^{2+}, 1 mM Co^{2+}, and 1 μM D600 (a methoxy derivative of verapamil). Extra Ca^{2+} was added to the medium of some cultures to attempt to increase Ca^{2+} influx. Since Ba^{2+} and Sr^{2+} may substitute for Ca^{2+} in some cellular processes, other cultures received these ions. Ca^{2+}, Ba^{2+}, and Sr^{2+} all were added at 5 mM, but, since precipitation occurred, the free ion concentrations were not known.

Chronic exposure to Mn^{2+} and Co^{2+} killed the neurons after several days. Additional Ca^{2+} and Mg^{2+} also significantly decreased cell survival, though the other agents tested had no obvious toxic effects. As shown in Table I, CM treatment increased the acetylcholine to catecholamine (ACh/CA) ratio more than 140-fold. Concurrent exposure to 20 mM K^+ lowered the ACh/CA ratio nearly 30-fold primarily by suppressing the development of the capacity for ACh production. Raising the Ca^{2+} concentration in the medium slightly enhanced the effect of depolarization with a 5-fold decrease in ACh synthesis. Ba^{2+} decreased the ratio about 2-fold, though the change was due to increased CA synthesis rather than decreased ACh synthesis. Sr^{2+} did not alter the ratio, though, like Ba^{2+}, it increased CA synthesis. Mg^{2+} and D600 both markedly increased the ACh/CA ratio. This effect was due to significant increases in ACh synthesis, which returned to CM-treated control levels in the Mg^{2+}-treated cultures. Thus, the two agents which can decrease Ca^{2+} influx, effectively reversed the influence of K^+ depolarization on transmitter choice, while addition of extra Ca^{2+} slightly potentiated the influence of depolarization.

Diphenylhydantoin (DPH) has multiple effects on neuronal physiology, including the ability to decrease Ca^{2+} influx (Goddard and Robinson, 1976; Pincus and Lee, 1976; Sohn and Ferrendelli, 1973). Table II shows the effect of DPH on neuronal transmitter choice. Addition of DPH in the presence of 20 mM K^+ counteracted the effects of depolarization. In fact, the ACh/CA ratio was higher than that of CM controls. ACh synthesis was increased and CA synthesis was decreased (though some of the CA decrease may be due to decreased neuronal growth). Thus, DPH appears to be another potent inhibitor of the ability of K^+ depolarization to influence transmitter choice. It should be noted that the concentration of DPH employed was somewhat toxic, resulting in decreased protein content (see Table IV) and total transmitter synthesis.

To further characterize the dependence of the developmental effect of elevated K^+ on calcium availability, neurons were grown in the presence of various concentrations of EGTA. Normal L15-CO_2 and 20 mM K^+ L15-CO_2 contain 1.25 mM calcium. Addition of 0.1 to 0.75 mM EGTA to the medium had no influence on the final ACh/CA ratio (Fig. 1). However, in 1.25 mM EGTA, the ACh/CA ratio increased 30-fold to 1.55. This ratio was also considerably higher than the 0.55 seen in CM-treated control cultures in this experiment. Some cultures were grown in 1.5 mM EGTA, but the neurons did not survive. Therefore, lowering Ca^{2+} with EGTA also inhibits the developmental effects of depolarization.

L15-CO_2 medium, CM, and calcium. Since the availability of calcium appears to be important in determining the extent of cholinergic induction in depolarized cultures, its role in transmitter choice in control and CM cultures was examined also (Table III).

Control cultures were primarily adrenergic with an ACh/CA ratio of 0.3, and CM increased ACh synthesis 43-fold. Additional Ca^{2+} did not affect ACh or CA synthesis. On the other hand, Ba^{2+} decreased the ratio in control medium and, in the presence of CM, markedly inhibited cholinergic induction, lowering both the ratio and ACh synthesis 25-fold. Mg^{2+} and D600 had effects on non-depolarized cultures, though the effects were

TABLE I

The role of calcium in the developmental effects of K^+ depolarization

Cultures were grown under uniform conditions for the first 2 days, 60% CM was added where indicated on day 9, and other additions were made on day 2. Neurons were counted on day 16 and transmitter assays were run on day 17 (see "Materials and Methods"). The KCl concentration in K^+ media was 20 mM; $CaCl_2$, $SrCl_2$, and $BaCl_2$ were added at 5 mM; $MgCl_2$ was added at 20 mM; and D600 was added at 1 μM. All data are expressed as the mean ± SEM for triplicate cultures. An analysis of variance was performed, and the groups were compared by the method of Scheffé (1959).

Additives	Neuronal Number	ACh/CA	ACh	CA
			fmol/cell	
None	3345 ± 375	0.04 ± 0.02[a]	0.1 ± 0.0[a]	2.6 ± 0.1
CM	3820 ± 240	5.68 ± 0.13	8.9 ± 0.8	1.6 ± 0.1
K^+ + CM	4220 ± 960	0.20 ± 0.06[a]	0.5 ± 0.2[a]	2.4 ± 0.4
K^+ + CM + Ca^{2+}	1025 ± 106[a]	0.07 ± 0.07[a]	0.1 ± 0.1[a]	1.8 ± 0.7
K^+ + CM + Sr^{2+}	2685 ± 49	0.21 ± 0.04[a]	1.0 ± 0.3[a]	5.4 ± 0.8[a, b]
K^+ + CM + Ba^{2+}	3180 ± 806	0.09 ± 0.03[a]	0.5 ± 0.2[a]	5.5 ± 0.3[a, b]
K^+ + CM + Mg^{2+}	1840 ± 520[a]	3.97 ± 0.64[a]	9.5 ± 0.4[b]	2.5 ± 0.3
K^+ + CM + D600	5170 ± 594	2.54 ± 0.42[a, b]	4.6 ± 0.9[a, b]	1.8 ± 0.2

[a] $p < 0.05$ compared to CM (line 2).
[b] $p < 0.05$ compared to K^+ + CM (line 3).

TABLE II

The effect of diphenylhydantoin on transmitter choice

Cultures were grown in the presence of elevated K^+ from day 2, 50% CM was added where indicated on day 8, and transmitter assays were performed on day 21. In this table, K^+ refers to 20 mM K^+ L15 medium and DPH refers to 0.4 mM diphenylhydantoin dissolved in dimethyl sulfoxide (DMSO) with 300 μl of DMSO/50 ml of medium. Each group contained triplicate cultures.

Additives	ACh/CA	ACh	CA
		pmol/culture	
None	0.05 ± 0.00	0.6 ± 0.1	12.2 ± 1.5
CM	1.09 ± 0.01[a]	9.0 ± 1.6[a, b]	8.2 ± 1.5
CM + DPH	4.32 ± 0.05[a]	3.9 ± 0.6	0.9 ± 0.2[a]
CM + K^+	0.11 ± 0.03	1.0 ± 0.3	9.3 ± 0.4
CM + K^+ + DPH	3.17 ± 0.13[a]	5.5 ± 1.8[a, b]	1.8 ± 0.6[a, b]

[a] $p < 0.05$ compared to control (line 1).
[b] $p < 0.05$ compared to CM + K^+ (line 4).

smaller than those under depolarizing conditions. Similarly, DPH further enhanced the cholinergic character of non-depolarized cultures, though the effect was due primarily to decreased CA synthesis (Table II). As will be discussed later, EGTA also enhanced the effectiveness of CM in non-depolarized cultures. Thus, in normal culture media, agents capable of affecting Ca^{2+} availability have effects similar to those seen in depolarizing medium. The exception is Ba^{2+}, which is a potent inhibitor of cholinergic induction in non-depolarizing medium (see "Discussion").

Calcium availability and the elevation of cAMP in depolarized neurons. The preceding paper demonstrated that depolarization leads to an increase in neuronal cAMP content (Walicke and Patterson, 1981). Since the availability of Ca^{2+} appears to be important for the effects of depolarization on transmitter choice, the effect of various Ca^{2+} uptake blockers on cAMP accumulation was examined (Table IV). As previously demonstrated, depolarization increases the content of cAMP by 25 to 100%. The addition of 20 mM Mg^{2+} or 1 μM D600 to the medium enabled the neurons to become cholinergic and reduced cAMP content to levels equal to, or lower than, the controls. On the other hand, in 1.25 mM EGTA,

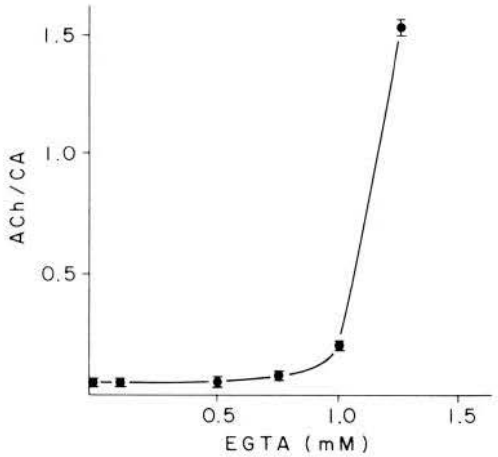

Figure 1. Effect of EGTA on cholinergic induction in 20 mM K^+ medium. Neurons were exposed to 20 mM K^+ and EGTA starting on day 2; 60% CM was added on day 11. Cultures were assayed for transmitter on day 21 as described. *Bars* give ±SEM for triplicate cultures.

TABLE III

The role of calcium in non-depolarizing media

Cultures were grown for 3 weeks in the presence of D600 or the ions indicated; 60% CM was present from day 11 where indicated. $CaCl_2$ and $BaCl_2$ were added at 5 mM and $MgCl_2$ at 20 mM; D600 was 1 μM. Each group contained triplicate cultures.

Additives	ACh/CA	ACh	CA
		pmol/culture	
None	0.31 ± 0.03[a]	1.0 ± 0.2[a]	3.0 ± 0.4
Ca^{2+}	0.40 ± 0.11[a]	1.6 ± 0.5[a]	3.9 ± 0.3
Ba^{2+}	0.06 ± 0.02[a]	0.3 ± 0.1[a]	5.1 ± 0.7
Mg^{2+}	1.21 ± 0.05[a]	8.8 ± 2.5[a]	7.3 ± 1.9
D600	0.85 ± 0.00[a]	4.6 ± 0.3[a]	5.4 ± 0.3
CM	9.15 ± 0.11	43.1 ± 5.3	4.7 ± 0.6
CM + Ca^{2+}	6.86 ± 1.14	31.6 ± 8.0	4.5 ± 0.4
CM + Ba^{2+}	0.41 ± 0.09[a]	1.7 ± 0.2[a]	4.3 ± 0.4
CM + Mg^{2+}	13.55 ± 1.58[a]	52.9 ± 3.8	3.6 ± 0.8
CM + D600	15.79 ± 0.11[a]	53.6 ± 10.0	3.5 ± 0.6

[a] $p < 0.05$ compared to CM control.

neurons became cholinergic, though their content of cAMP was still elevated in comparison to the control. The effects of DPH on cAMP were examined in Experiment 2. Dimethyl sulfoxide (DMSO), the vehicle for solubilizing DPH, did not affect transmitter choice or cAMP levels. Cultures in DPH became quite cholinergic, more so than the controls, but cAMP levels were even higher than those in K^+-depolarized cultures. In other experiments, addition of DPH did not alter significantly cAMP levels in depolarized cultures while still exerting a cholinergic influence (data not shown). Thus, two of the agents which reversed the effect of depolarization on transmitter choice, Mg^{2+} and D600, were able to decrease the levels of cAMP seen in depolarized cells. However, EGTA and DPH also reversed the effect of depolarization on cholinergic induction but did not affect cAMP accumulation in depolarized cells.

Since veratridine also affects neuronal transmitter choice (Walicke et al., 1977), the relationship of Ca^{2+} availability and cAMP levels also were examined with this agent (Table V). Exposure to 1 μg/ml (1.5 μM) veratridine increased cAMP levels about 3-fold and lowered the ACh/CA ratio about 10-fold. Again, 20 mM Mg^{2+}, D600, and EGTA reversed the effects of depolarization on the transmitter ratio, though D600 was less effective than the other agents. D600 and Mg^{2+} both decreased cAMP to levels comparable to those in nondepolarized control cultures. EGTA had relatively little effect on cAMP levels, though the final result was significantly different from the values seen in either control or depolarized cultures. These results with veratridine are similar to the observations made on the cultures depolarized by elevated K^+.

The role of released transmitter. The neurons in these cultures make numerous synapses with each other under both adrenergic and cholinergic conditions (Johnson et al., 1976; Landis, 1980). The requirement for Ca^{2+} in the developmental actions of depolarization could indicate that a transmitter released at such synapses is mediating the effects of depolarization on transmitter choice and cAMP accumulation. However, blocking both α- and β-adrenergic receptors with propranolol and phenoxybenzamine did not alter transmitter synthesis or cAMP levels (Table VI). Adenosine 3′:5′-triphosphate (ATP) is probably released from sympathetic neurons along with CA (Su et al., 1971) and could be hydrolyzed by extracellular enzymes to adenosine which in turn could stimulate adenosine receptors on the neurons. Therefore, adenosine deaminase and alloxazine were used to eliminate this possibility. The concentration of deaminase used was adequate to reverse the effects of added 1 mM adenosine on transmitter choice (data not shown). Neither the deaminase nor alloxazine affected the ACh/CA ratio, and the deaminase did not affect cAMP. The effects of theophylline, another adenosine receptor antagonist, were discussed in the preceding paper (Walicke and Patterson, 1981). In other experiments, atropine (1 μM), hexamethonium (1 mM), and Dibenamine (10 μM) also were observed to have no effect on the transmitter choice of depolarized neurons (data not shown).

Cyclic nucleotide addition and calcium. Addition of cyclic nucleotide derivatives can inhibit cholinergic differentiation in the presence of CM (Walicke and Patterson, 1981). The role of calcium in this phenomenon was investigated using 1.25 mM EGTA (Table VII). The neurons in this experiment, which were not depolarized, had more difficulty surviving in medium with low extracellular Ca^{2+}. EGTA had to be removed from the medium on days 7 and 12 *in vitro* to allow neuronal survival. CM and cyclic nucleotides also were removed with the EGTA so that all influences on transmitter choice were presented only with low extracellular Ca^{2+}. Despite these exposures to Ca^{2+}, most cultures grown in EGTA had noticeably lower protein contents than the controls.

In this experiment, all cultures were exposed to 50% CM. As previously observed, dibutyryl cAMP (dbcAMP), dibutyryl cGMP (dbcGMP), and prostaglandin E_1 (PGE_1) all lowered ACh synthesis significantly. With the addition of EGTA, all of the cultures were markedly more cholinergic than their controls by the criterion of the ACh/CA ratio. Even cultures in CM alone and EGTA

TABLE IV

Effect of Ca^{2+} on cAMP in K^+-depolarized neurons

Cultures received the additives shown on day 2 and were harvested for either transmitter or cyclic nucleotide and protein assays on day 14. Cultures in the first experiment received an ammonium sulfate precipitate of CM at the equivalence of 100% CM; cultures in the second experiment received 50% CM. K^+ was 20 mM; D600 was 1 μM; EGTA was 1.25 mM; DMSO was added at 300 μl/55 ml of medium; DPH was dissolved in DMSO and added to 0.4 mM. Figures with ±SEM are for duplicate determinations; others are single determinations.

Additives	ACh/CA	cAMP	Protein
		fmol/μg	*μg*
	Experiment 1		
CM	1.54 ± 0.04	8.3 ± 0.4[a]	19.4 ± 1.4
CM + K^+	0.02 ± 0.00	16.5 ± 2.0	12.8 ± 0.0
CM + K^+ + Mg^{2+}	2.37	7.6 ± 1.5[a]	20.0 ± 1.4
CM + K^+ + D600	1.46 ± 0.15	4.0 ± 0.7[a]	22.0 ± 0.0
CM + K^+ + EGTA	4.42 ± 0.04[a]	11.8 ± 0.2	23.9 ± 0.4
	Experiment 2		
CM	0.46 ± 0.02	3.4 ± 0.1	49.8 ± 2.5
CM + K^+	0.07 ± 0.02[b]	4.2 ± 0.2	65.3 ± 0.4
CM + K^+ + DMSO	0.09 ± 0.02[b]	5.1 ± 1.4	46.0 ± 0.0
CM + K^+ + DMSO + DPH	1.28 ± 0.01[b]	9.9 ± 1.0[b]	16.5 ± 4.2[b]

[a] $p < 0.05$ compared to CM + K^+ (Experiment 1, line 2).
[b] $p < 0.05$ compared to control (Experiment 2, line 1).

TABLE V

Effect of Ca^{2+} on cAMP in veratridine-depolarized cultures

Cultures received the additives shown and 50% CM on day 2 and were assayed on day 14. Veratridine was added at a concentration of 1 μg/ml (1.5 μM), Mg^{2+} at 20 mM, D600 at 1 μM, and EGTA at 1.25 mM. Figures are for duplicate determinations except the ACh/CA ratio on line 5, which is a single determination.

Additives	ACh/CA	cAMP
		fmol/μg
CM	1.45 ± 0.85[a]	8.04 ± 0.3[a]
CM + veratridine	0.13 ± 0.07	26.0 ± 3.0
CM + veratridine + Mg^{2+}	1.35 ± 0.08[a]	7.1 ± 1.9[a]
CM + veratridine + D600	0.53 ± 0.08	10.6 ± 0.6[a]
CM + veratridine + EGTA	1.96[a]	17.7 ± 1.4[a, b]

[a] $p < 0.05$ compared to CM + veratridine (line 2).
[b] $p < 0.05$ compared to control (line 1).

TABLE VI

Transmitter receptors and the developmental effect of K^+ depolarization

Cultures received the additives shown and 50% CM starting on day 2 and they were harvested on day 14. The K^+ concentration was 20 mM; propranolol and phenoxybenzamine were added at 10 μM, adenosine deaminase at 15 units/ml, and alloxazine at 1 mM. Each group contained triplicate or duplicate cultures.

Additives	ACh/CA	ACh	CA	cAMP
		pmol/dish		*fmol/μg protein*
CM	0.79 ± 0.02	5.17 ± 1.03	6.6 ± 1.5	4.86 ± 0.09
CM + K^+	0.02 ± 0.01[a]	0.60 ± 0.04[a]	26.0 ± 7.0	6.74 ± 0.23[b]
CM + K^+ + propranolol + phenoxybenzamine	0.02 ± 0.01[a]	0.66 ± 0.11[a]	28.9 ± 8.1	6.87 ± 0.56[b]
CM + K^+ + adenosine deaminase	0.02 ± 0.00[a]	0.55 ± 0.01[a]	27.9 ± 2.7	7.32 ± 0.68[a]
CM + K^+ + alloxazine	0.03 ± 0.01[a]	0.84 ± 0.20[a]	34.4 ± 0.1[a]	

[a] $p < 0.05$ compared to control (line 1).
[b] $p < 0.1$ compared to control (line 1).

TABLE VII

Exogenous cyclic nucleotides and EGTA

Cultures received the additives shown and 50% CM starting on day 2 and were harvested on day 14. All cultures were placed in normal L15-CO_2 and grown in medium without CM or additives for 24 hr on days 7 and 12. The EGTA concentration was 1.25 mM; dbcAMP, 1 mM; dbcGMP, 1 mM; PGE_1, 50 μM. All cultures receiving dbcAMP, dbcGMP, or PGE_1 also received 10 μM RO 20-1724. Each figure represents duplicate determinations except for cAMP and protein in line 2 which were single determinations.

Additives	ACh/CA	ACh	CA	cAMP	Protein
		pmol/dish		*fmol/μg protein*	*μg/dish*
CM	5.54 ± 0.86	46.1 ± 2.96	8.46 ± 1.85	7.05 ± 0.49	12.8 ± 0.8
CM + EGTA	13.54 ± 1.76[a]	11.4 ± 7.36[a]	0.81 ± 0.44[a]	4.1	4.4
CM + dbcAMP	1.69 ± 0.13	21.4 ± 1.44[b]	12.64 ± 0.13		12.0 ± 2.0
CM + dbcAMP + EGTA	7.54 ± 1.20[a, c]	28.5 ± 1.47	3.81 ± 0.41[a]		7.5 ± 1.3
CM + dbcGMP	1.94 ± 0.18	16.3 ± 1.70[b]	8.41 ± 0.08	26.0 ± 1.98[b]	12.4 ± 1.4
CM + dbcGMP + EGTA	11.51 ± 2.30[a]	4.1 ± 0.15[b]	0.36 ± 0.09[a]	27.6 ± 4.60[b]	5.0 ± 0.6
CM + PGE_1	0.40 ± 0.06[b]	5.7 ± 0.13[b]	14.50 ± 2.44[b]	20.3 ± 3.60[b]	14.2 ± 1.1
CM + PGE_1 + EGTA	3.20 ± 0.23[c]	27.1 ± 5.88[a]	8.45 ± 1.22[a, c]	16.3 ± 1.22	13.7 ± 4.9

[a] $p < 0.05$ compared to own control without EGTA.
[b] $p < 0.1$ compared to CM control (line 1).
[c] $p < 0.1$ compared to CM + EGTA (line 2).

had a roughly 2-fold increase in the ACh/CA ratio. Cultures in EGTA plus dbcAMP or PGE_1 did not become as cholinergic as the cultures in EGTA plus CM alone by the criterion of the ACh/CA ratio, though EGTA appeared to overcome completely the effects of dbcGMP. Unfortunately, it is difficult to compare the changes in ACh and CA synthesis because of the decreased protein and total transmitter contents. Only the cultures in PGE_1 plus EGTA contained significantly more ACh than their controls, but these were the only EGTA cultures without a decline in protein content. In each case, there was a significant decline in CA synthesis, which could reflect partly the lowered protein content. Although the results are complicated by the toxicity problem, EGTA does appear to reverse largely the effects of cyclic nucleotides and PGE_1 on transmitter choice.

As shown in Table VII, dbcGMP increased cAMP about 3.5-fold and PGE_1 increased it about 3-fold. EGTA did not lower cAMP content in neurons exposed to dbcGMP and only slightly affected that of neurons grown in PGE_1. The most striking result of this experiment was that EGTA did not interfere with the ability of these effectors to increase intracellular cAMP. Thus, exposure to EGTA can uncouple the increase in cAMP content from changes in the transmitter choice of the developing neurons.

Discussion

The experiments presented in this paper demonstrate that the availability of calcium has significant effects on the neurotransmitter choice of developing sympathetic neurons. Depolarization by either elevated K^+ or veratridine cannot direct the neurons toward completing adrenergic differentiation unless Ca^{2+} is available. Even neurons grown in normal L15-CO_2 or CM become more cholinergic when the extracellular concentration of free Ca^{2+}, or its influx, is lowered. The developmental response of the neurons to dibutyryl cyclic nucleotides and effectors of intracellular cAMP also is reduced markedly in medium with a lowered Ca^{2+} concentration. Ca^{2+} influx also may be involved in the elevation of cAMP levels which are seen in depolarized neurons. The evidence suggests an important role for Ca^{2+} in the processes involved in directing the choice of neurotransmitter during development and is consistent with a role for Ca^{2+} as the primary mediator of the effects of depolarization.

Calcium and neurotransmitter choice. Several different agents were used to demonstrate a role for Ca^{2+} in the developmental effects of depolarization: 20 mM Mg^{2+}, D600, EGTA, and DPH. The similarity of the effects of these disparate agents strengthens the hypothesis that Ca^{2+} influx underlies the effects of depolarization. All of

these agents, however, may have additional effects on neuronal metabolism, especially during the prolonged exposures used in these experiments. Mg^{2+}, along with other bi- and trivalent ions (Co^{2+}, Mn^{2+}, Ln^{3+}), has long been recognized to block Ca^{2+} influx through voltage-dependent channels (McAfee and Yarowsky, 1979; Horn and McAfee, 1980; O'Lague et al., 1978), though Mg^{2+} itself also functions as an important ion required in the activity of many cellular enzymes. The alkaloid, D600, may be relatively specific for voltage-dependent Ca^{2+} channels in some excitable tissues (Kohlhardt et al., 1972; Haeusler, 1972) but has also been reported to affect "slow" Na^+ current (Shigenobu et al., 1974), K^+ conductance (Kass and Tsien, 1975; Nawrath et al., 1977), Mg^{2+} extrusion (De Weer, 1976), and transmitter re-uptake systems (McGee and Schneider, 1979). Besides its effects on Ca^{2+} influx (Goddard and Robinson, 1976; Pincus and Lee, 1976; Sohn and Ferrendelli, 1973) and post-tetantic potentiation (Raines and Standaert, 1966), DPH has been reported to affect Na^+ conductance (Lipicky et al., 1972; Raines and Standaert, 1966) and the activity of the (Na^+,K^+)-ATPase (DenHertog, 1972). It is interesting that DPH has been observed to inhibit Ca^{2+}-dependent protein kinases (DeLorenzo, 1977). The potent effects of DPH on transmitter choice, despite its rather poor efficacy as a Ca^{2+} influx inhibitor, suggest the possibility that DPH could be working through a kinase in this system. EGTA directly reduces the concentration of free extracellular Ca^{2+}. Measurements with a calcium-sensitive electrode indicated that about 1 to 10 μM free Ca^{2+} remained in the medium in the presence of 1.25 mM EGTA (M. Goy and R. Harris-Warrick, personal communication). Although, of all the agents used, EGTA may have the most straightforward effect on Ca^{2+} influx, it remains possible that EGTA may enter the cells during these long exposures and directly affect intracellular Ca^{2+} metabolism.

The effects of Ca^{2+} availability on neuronal development are not limited to depolarized cultures but can also be seen in neurons grown in normal L15-CO_2 or in CM. Mg^{2+}, D600, EGTA, and DPH all increase the ACh/CA ratio in cultures which are not depolarized (Tables II, III, and VII). In these non-depolarized cultures, Ba^{2+} caused marked suppression of cholinergic induction. Although this could be a specific effect of the ion on neuronal development, more likely it reflects the ability of Ba^{2+} to decrease resting K^+ conductance and lead to neuronal depolarization (McLachlan, 1977).

It is possible that the effects of these Ca^{2+} blocking agents on neuronal transmitter choice reflect a hyperpolarization caused by blockade of Ca^{2+} channels. Although the physiological effects of these agents have not been investigated in this system, low extracellular Ca^{2+} (as in the presence of EGTA) is widely recognized to lower action potential threshold with relatively little effect on membrane potential, thus markedly increasing spontaneous neuronal activity (Frankenhauser and Hodgkin, 1957; McLachlan, 1977). It appears unlikely, therefore, that hyperpolarization and the cessation of neuronal activity underlie the developmental effects of Ca^{2+} deprivation.

The effects of Ca^{2+} availability on neurotransmitter choice could indicate that the main reason that more of the sympathetic neurons become cholinergic *in vitro* than *in vivo* is that there is not an optimal concentration of Ca^{2+} present in the usual culture medium. However, addition of more Ca^{2+} to the medium does not change the final ACh/CA ratio in non-depolarized cultures (Table III) and has only a slight effect in depolarized cultures (Table I). It is still possible that cultured neurons may regulate their cellular content of Ca^{2+} differently than those *in vivo*, causing increased susceptibility to cholinergic induction. These results could suggest also that the CM factor influences transmitter development by influencing the entry of Ca^{2+} into the neurons. Investigation of this interesting possibility will require direct studies of Ca^{2+} fluxes.

Calcium and cyclic nucleotides. Depolarization of the neurons by elevated K^+ or veratridine leads to an increase in cAMP content, and Mg^{2+} and D600 can inhibit this increase, suggesting that Ca^{2+} influx may be involved in the effect on cAMP. On the other hand, neither EGTA nor DPH interferes with cAMP accumulation during depolarization. These contradictory observations may reflect the differences between the agents on other facets of cellular metabolism which may be involved in the eventual elevation of cAMP. The results are equivocal on the possible role of Ca^{2+} influx in the depolarization-induced increase in cAMP, and more thorough studies will be required to examine a possible relationship.

Direct electrical stimulation and depolarization have been reported previously to increase cAMP levels in sympathetic ganglia (McAfee et al., 1971) and brain slices (Kakiuchi et al., 1969). This increase in cAMP often is attributed to the release of various neurotransmitters, particularly dopamine (Kalix et al., 1974), norepinephrine (Williams and Rodnight, 1974), or adenosine (Sattin and Rall, 1970), followed by stimulation of postsynaptic adenylate cyclase-linked receptors. In the present experiments, the requirement for Ca^{2+} in the stimulation of cAMP accumulation also could indicate the action of an interposed neurotransmitter. However, blockade of α- and β-adrenergic, muscarinic, nicotinic, and adenosine receptors did not interfere with the actions of depolarization on either cAMP accumulation or transmitter choice. The neurons do not synthesize detectable γ-aminobutyric acid (GABA), serotonin, or histamine (Mains and Patterson, 1973) so that none of these recognized transmitters should be involved. Of course, an unrecognized transmitter or modulator could play a role, but it is hypothesized that depolarization and Ca^{2+} entry themselves are directly responsible for the effects under study. Interestingly, Roch and Kalix (1975) obtained evidence of a direct depolarization-mediated increase in cAMP in the bovine superior cervical ganglion, which may be localized to presynaptic terminals.

Calcium and cyclic AMP as second messengers. As summarized in the previous paper, the evidence concerning a possible role for cAMP as second messenger in the effects of depolarization on transmitter choice was equivocal. The influence of exogenous cyclic nucleotides on transmitter choice and the elevation of cAMP by depolarization supported the hypothesis. However, several points of evidence argued against such a role for cAMP,

particularly the finding that theophylline uncoupled the increase in cAMP from later changes in development. In the present paper, it has been shown further that EGTA and DPH also can uncouple the increase in cAMP caused by depolarization from the eventual change in transmitter function. The action of these agents argues against a role for cAMP as the second messenger normally involved in the developmental effects of depolarization.

Evidence consistent with a role for Ca^{2+} as the chief agent coupling depolarization to later changes in development has been presented in this paper: Several disparate Ca^{2+} influx blockers can affect transmitter choice. The nature of the three agents capable of uncoupling cAMP accumulation from developmental changes also points to a possible role for Ca^{2+}. As discussed above, EGTA and DPH are known to affect Ca^{2+} influx and Ca^{2+}-dependent phosphokinases, so that their effect on development is likely to be secondary to effects on Ca^{2+}. Theophylline remains more difficult to understand. It is unlikely to favor cholinergic development through its activity as a phosphodiesterase inhibitor or an adenosine receptor inhibitor, based on the other data presented. Acute exposures to millimolar concentrations of methylxanthines have been reported to depolarize some sympathetic neurons (Kuba and Nishi, 1976) and to hyperpolarize others by increasing K^+ conductance and the activity of the (Na^+,K^+)-ATPase (Kuba, 1980; Skok et al., 1978). The latter mechanism of hyperpolarization could function in K^+-depolarized neurons and might explain the effects on transmitter choice.

Finally, theophylline is recognized to interfere with the storage of Ca^{2+} in sarcoplasmic-like cellular stores (Johnson and Inesi, 1969) which have been observed recently in neurons (Blaustein et al., 1978; Kuba, 1980). Initially, this would be expected to lead to increased cytoplasmic Ca^{2+}, though more prolonged exposures have been reported to lead to Ca^{2+} depletion after loss of the intracellular stores (Ito and Kuriyama, 1971; Pfaffman and McFarland, 1978; Weber and Herz, 1968). Therefore, it is possible that theophylline also is acting on cellular Ca^{2+} metabolism to uncouple cAMP from eventual changes in development. Although more direct studies of Ca^{2+} metabolism will be necessary, it appears possible that all three agents capable of uncoupling cAMP from changes in transmitter choice could work by affecting Ca^{2+}.

It was observed in the present study that restricting Ca^{2+} availability with EGTA decreased the effects of dibutyryl cyclic nucleotides and effectors of intracellular cAMP on development. Cyclic AMP levels were not themselves affected. These observations could be interpreted as indicating that exogenously added cyclic nucleotides act, at least in part, by increasing Ca^{2+} influx. However, EGTA only partially reverses the effects of dbcAMP and PGE_1 on transmitter choice, which is consistent with the interpretation that both Ca^{2+} and cAMP can influence transmitter choice independently. For example, it is possible that both Ca^{2+} and cAMP independently and additively affect the phosphorylation of a particular target protein involved in control of gene expression. Some overlap in target proteins for these phosphokinase systems has been demonstrated in brain (Sieghart et al., 1979). Distinguishing these possibilities will require more thorough studies of the relationships between cellular cAMP and Ca^{2+} metabolism in sympathetic neurons.

The experiments in this and the preceding paper (Walicke and Patterson, 1981) indicate that a variety of signals can influence the transmitter choice of sympathetic neurons maintained in culture, including a factor in CM, electrical activity, agents which elevate cAMP, and butyrate. Hormones also can influence the choice (McLennan et al., 1980); however, they appear not to act directly on the neurons, but rather indirectly, by affecting the production or release of the CM factor by the nonneuronal cells (Fukada, 1980). It appears that the ability of these neurons to respond to the differentiation signal in CM is counteracted by a variety of "maturation" signals which appear to be agents capable of increasing either cAMP and/or neuronal Ca^{2+}. At present, it is not possible to determine whether cAMP and Ca^{2+} act independently or by altering each other's metabolism. In the case of depolarization or neuronal activity, however, it appears that Ca^{2+} is a major mediator of the eventual effects on transmitter choice. This hypothesis is attractive because the same ion fluxes which constitute normal neuronal activity also could be employed to mediate developmental decisions.

References

Black, I., I. Hendry, and L. Iversen (1971) Transsynaptic regulation of growth and development of adrenergic neurones in a mouse sympathetic ganglion. Brain Res. *34:* 229–240.

Blaustein, M. (1976) Barbiturates block calcium uptake by stimulated and potassium-depolarized rat sympathetic ganglia. J. Pharmacol. Exp. Ther. *196:* 80–86.

Blaustein, M., R. Ratzlaff, and E. Schweitzer (1978) Calcium buffering in presynaptic nerve terminals. II. Kinetic properties of the non-mitochondrial Ca sequestration mechanism. J. Gen. Physiol. *72:* 43–66.

Cheung, W. (1980) Calmodulin plays a pivotal role in cellular regulation. Science *207:* 19–27.

DeLorenzo, R. (1977) Antagonistic action of diphenylhydantoin and calcium on the level of phosphorylation of particular rat and human brain proteins. Brain Res. *134:* 125–138.

DenHertog, A. (1972) The effect of diphenylhydantoin on the electrogenic component of the sodium pump in mammalian non-myelinated nerve fibres. Eur. J. Pharmacol. *19:* 94–97.

De Weer, P. (1976) Axoplasmic free magnesium levels and magnesium extrusion from squid giant axons. J. Gen. Physiol. *68:* 159–178.

Frankenhauser, B., and A. Hodgkin (1957) The action of calcium on the electrical properties of squid axons. J. Physiol. (Lond.) *137:* 218–244.

Fukada, K. (1980) Hormonal control of neurotransmitter choice in sympathetic neuron cultures. Nature *287:* 553–555.

Goddard, G., and J. Robinson (1976) Uptake and release of calcium by rat brain synaptosomes. Brain Res. *110:* 331–350.

Haeusler, G. (1972) Differential effect of verapamil on excitation-contraction coupling in smooth muscle and on excitation-secretion coupling in adrenergic nerve terminals. J. Pharmacol. Exp. Ther. *180:* 672–682.

Horn, J., and D. McAfee (1980) Alpha-adrenergic inhibition of calcium-dependent potentials in rat sympathetic neurons. J. Physiol. (Lond.) *301:* 191–204.

Ito, Y., and H. Kuriyama (1971) Caffeine and excitation-contraction coupling in the guinea pig *Taenia coli.* J. Gen. Physiol. *57:* 448–463.

Johnson, M., D. Ross, M. Meyers, R. Rees, R. Bunge, E. Wakshull, and H. Burton (1976) Synaptic vesicle cytochemistry changes when cultured sympathetic neurons develop cholinergic interactions. Nature *262:* 308–310.

Johnson, P., and G. Inesi (1969) The effect of methylxanthines and local anaesthetics on fragmented sarcoplasmic reticulum. J. Pharmacol. Exp. Ther. *169:* 308–314.

Kakiuchi, S., T. Rall, and H. McIlwain (1969) The effect of electrical stimulation upon the accumulation of adenosine 3′,5′-phosphate in isolated cerebral tissue. J. Neurochem. *16:* 485–491.

Kalix, P., D. McAfee, M. Schorderet, and P. Greengard (1974) Pharmacological analysis of synaptically mediated increase in cyclic adenosine monophosphate in rabbit superior cervical ganglion. J. Pharmacol. Exp. Ther. *188:* 676–687.

Kass, R., and R. Tsien (1975) Multiple effects of calcium antagonists on plateau currents in cardiac Purkinje fibers. J. Gen. Physiol. *66:* 169–192.

Kohlhardt, M., B. Bauer, H. Krause, and A. Fleckenstein (1972) Differentiation of the transmembrane Na and Ca channels in mammalian cardiac fibres by the use of specific inhibitors. Pflügers Arch. *335:* 309–322.

Kuba, K. (1980) Release of calcium ions linked to the activation of potassium conductance in a caffeine-treated sympathetic neurone. J. Physiol. (Lond.) *298:* 251–269.

Kuba, K., and S. Nishi (1976) Rhythmic hyperpolarization and depolarization of sympathetic ganglion cells induced by caffeine. J. Neurophysiol. *39:* 547–563.

Landis, S. (1980) Developmental changes in the neurotransmitter properties of dissociated sympathetic neurons: A cytochemical study of the effect of medium. Dev. Biol. *77:* 349–361.

Lipicky, R., D. Gilbert, and I. Stillman (1972) Diphenylhydantoin inhibition of sodium conductance in squid giant axon. Proc. Natl. Acad. Sci. U. S. A. *69:* 1758–1760.

Mains, R., and P. Patterson (1973) Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. J. Cell Biol. *59:* 329–345.

McAfee, D., and P. Yarowsky (1979) Calcium-dependent potentials in the mammalian sympathetic neurone. J. Physiol. (Lond.) *290:* 507–523.

McAfee, D., M. Schorderet, and P. Greengard (1971) Adenosine 3′,5′-monophosphate in nervous tissue: Increase associated with synaptic transmission. Science *171:* 1156–1158.

McGee, R., and J. Schneider (1979) Inhibition of high affinity synaptosomal uptake systems by verapamil. Mol. Pharmacol. *16:* 877–885.

McLachlan, E. (1977) The effects of strontium and barium ions at synapses in sympathetic ganglia. J. Physiol. (Lond.) *267:* 497–518.

McLennan, I., C. Hill, and I. Hendry (1980) Glucocorticosteroids modulate transmitter choice in developing superior cervical ganglion. Nature *283:* 206–207.

Means, A., and J. Dedman (1980) Calmodulin—An intracellular calcium receptor. Nature *285:* 73–77.

Nawrath, H., R. TenEick, T. McDonald, and W. Trautwein (1977) On the mechanism underlying the action of D600 on slow inward current and tension in mammalian myocardium. Circ. Res. *40:* 408–414.

O'Lague, P., D. Potter, and E. Furshpan (1978) Studies on rat sympathetic neurons developing in cell culture. I. Growth characteristics and electrophysiological properties. Dev. Biol. *67:* 384–403.

Pfaffman, M., and S. McFarland (1978) Relationship between theophylline-induced relaxation and excitation-contraction coupling in intestinal smooth muscle. Arch. Int. Pharmacodyn. Ther. *232:* 180–191.

Pincus, J., and S. Lee (1976) Diphenylhydantoin and calcium. Arch. Neurol. *29:* 239–244.

Raines, A., and F. Standaert (1966) Pre- and postjunctional effects of diphenylhydantoin at the cat soleus neuromuscular junction. J. Pharmacol. Exp. Ther. *153:* 361–366.

Rasmussen, H., and D. Goodman (1977) Relationships between calcium and cyclic nucleotides in cell activation. Physiol. Rev. *57:* 422–509.

Robinson, J., and E. Kabela (1977) Distribution of ^{45}Ca in the superior cervical ganglion of the cat. J. Neurobiol. *8:* 511–522.

Roch, P., and P. Kalix (1975) Adenosine 3′,5′-monophosphate in bovine superior cervical ganglion: Effect of high extracellular potassium. Biochem. Pharmacol. *24:* 1293–1296.

Sattin, A., and T. Rall (1970) The effect of adenosine and adenine nucleotides on the cyclic adenosine 3′,5′-phosphate content of guinea pig cerebral cortex slices. Mol. Pharmacol. *6:* 13–23.

Scheffé, H. (1959) *Analysis of Variance*, John Wiley, New York.

Shigenobu, K., J. Schneider, and N. Sperelakis (1974) Verapamil blockade of slow Na^+ and Ca^{2+} responses in myocardial cells. J. Pharmacol. Exp. Ther. *190:* 280–288.

Sieghart, W., J. Forn, and P. Greengard (1979) Ca^{2+} and cyclic AMP regulate phosphorylation of the same two membrane-associated proteins specific to nerve tissue. Proc. Natl. Acad. Sci. U. S. A. *76:* 2475–2479.

Skok, V., N. Storch, and S. Nishi (1978) The effect of caffeine on the neurons of a mammalian sympathetic ganglion. Neuroscience *3:* 697–708.

Sohn, R., and J. Ferrendelli (1973) Inhibition of Ca^{2+} transport into rat brain synaptosomes by diphenylhydantoin. J. Pharmacol. Exp. Ther. *185:* 272–275.

Stallcup, W. (1979) Sodium and calcium fluxes in a clonal cell line. J. Physiol. (Lond.) *286:* 525–540.

Su, C., J. Bevan, and G. Burnstock (1971) ^3H-Adenosine triphosphate: Release during stimulation of enteric nerves. Science *173:* 336–338.

Walicke, P. A., and P. H. Patterson (1981) On the role of cyclic nucleotides in the transmitter choice made by cultured sympathetic neurons. J. Neurosci. *1:* 333–342.

Walicke, P., R. Campenot, and P. Patterson (1977) Determination of transmitter function by neuronal activity. Proc. Natl. Acad. Sci. U. S. A. *74:* 5767–5771.

Weber, A., and R. Herz (1968) The relationship between caffeine contracture of intestinal muscle and the effect of caffeine on the reticulum. J. Gen. Physiol. *52:* 750–759.

Williams, M., and R. Rodnight (1974) Evidence for a role for protein phosphorylation in synaptic function in the cerebral cortex mediated through a β-noradrenergic receptor. Brain Res. *77:* 502–506.